Mechanism of bisphosphonate-related osteonecrosis of the jaw (BRONJ) revealed by targeted removal of legacy bisphosphonate from jawbone using equilibrium competing inert hydroxymethylene diphosphonate

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Abstract:
Bisphosphonate-related osteonecrosis of the jaw (BRONJ) presents as a morbid jawbone lesion in patients exposed to a nitrogen-containing bisphosphonate (N-BP). Although it is rare, BRONJ has caused apprehension among patients and healthcare providers and decreased acceptance of this anti-resorptive drug class to treat osteoporosis and metastatic osteolysis. We report here a novel method to elucidate the pathological mechanism of BRONJ by the selective removal of legacy N-BP from the jawbone using an intra-oral application of hydroxymethylene diphosphonate (HMDP) formulated in liposome-based deformable nanoscale vesicles (DNV). After maxillary tooth extraction, zoledronate-treated mice developed delayed gingival wound closure, delayed tooth extraction socket healing and increased jawbone osteonecrosis consistent with human BRONJ lesion. Single cell RNA sequencing of mouse gingival cells revealed oral barrier immune dysregulation and unresolved pro-inflammatory reaction. HMDP-DNV topical applications to nascent mouse BRONJ lesions resulted in accelerated gingival wound closure and bone socket healing as well as attenuation of osteonecrosis development. The gingival single cell RNA sequencing demonstrated resolution of chronic inflammation by increased anti-inflammatory signature gene expression of lymphocytes and myeloid-derived suppressor cells. This study suggests that BRONJ pathology is related to N-BP levels in jawbones and demonstrates the potential of HMDP-DNV as an effective BRONJ therapy.

Brief Summary
The targeted removal of legacy bisphosphonate from the jawbone by competitive equilibrium therapy not only elucidated the pathological mechanism of bisphosphonate-related osteonecrosis of the jaw (BRONJ) but also established a highly translatable therapeutic option for BRONJ.
INTRODUCTION

Nitrogen-containing bisphosphonates (N-BPs) are prototypical anti-resorptive agents (1-3) initially marketed to prevent bone fractures and to treat osteopenia or osteoporosis (4, 5). N-BPs have been widely prescribed for postmenopausal women who have a bone mineral density T score of -2.5 or less, a history of spine or hip fracture, or a Fracture Risk Assessment Tool score (6) indicating increased fracture risk. N-BP treatment with an increased dose and frequency was also given to patients with multiple myeloma (7) or metastatic cancers to bone (8, 9) to address tumor-induced osteolysis, hypercalcemia and bone pain. N-BPs are well tolerated, exhibit few side-effects, and have established clinical benefits (10).

In the 2000’s, cases of osteonecrosis in the jawbone (ONJ) emerged among a minority of patients with a history of N-BP treatment, usually occurring at a dental infection site (11) or after dentoalveolar surgery such as a tooth extraction (12, 13). Originally designated “Bisphosphonate-related ONJ” (BRONJ), this syndrome has also proven to be associated with non-bisphosphonate antiresorptive agents such as denosumab, a humanized anti-RANKL monoclonal antibody and anti-angiogenesis drugs. As a result, the American Association for Oral & Maxillofacial Surgeons (AAOMS) has proposed the term “Medication-related ONJ” (MRONJ) reflecting the association of ONJ with a multiplicity of antiresorptive agents (14). Incidents of MRONJ reported to the United States Food and Drug Administration (FDA)’s Adverse Event Reporting System (FAERS) peaked from the first quarter of 2010 to the first quarter of 2014 with approximately 30,000 cases during this period, among which BRONJ represented the major fraction (15). The FARES database may underreport the incidents because of variations in provider’s perceptions of how severe an event needs to be to warrant submission of the event report (16). We present an alternative MRONJ estimation of approximately 36,000
new cases per year in the US (Table 1). Considering the long cure period, the cumulative MRONJ patient number may exceed this annual estimation; however, it is highly conceivable that MRONJ is a rare disease defined by the FDA’s orphan disease (17).

Typical disease symptoms include gingival dehiscence and exposure of necrotic jawbone (14, 18, 19), which often becomes infected, resulting in pain, erythema, and purulent drainage (20). The extent of a BRONJ lesion has been measured for the absolute or relative open wound area and the time taken for mucosa to completely cover necrotic tissue and exposed bone (“cure period”) (21). A multicenter case registry study reported either resolution (35%) or improvement (10%) in 207 evaluable BRONJ patients within the study period of 2 years with conservative treatments such as irrigation and antibiotic medications (22). However, 37% of patients did not respond to the conventional treatment and exhibited either progression or stable conditions. Although the reported case incident is small, long-lasting severe clinical symptoms of MRONJ in some patients have created apprehension among patients and healthcare providers (23, 24).

The NIH Workshop on “Pathway to Prevention (P2P) for Osteoporotic Fracture” in 2018 highlighted the acute need of research to mitigate serious adverse events such as MRONJ and atypical femur fracture to prevent the increasing threat of osteoporotic fractures (25). The P2P workshop particularly highlighted the markedly decreased acceptance of anti-resorptive medications by osteoporosis patients, despite the significant benefit of these drugs for this indication (26). The recent decline in N-BP prescription, reflecting diminished patient acceptance of these drugs, has been linked to a statistical rise in bone-related complications (27). Due to the long-lasting half-life of N-BPs once chemisorbed to bone, the discontinuation of N-BPs and switching to other anti-resorptive medications may still pose a risk of developing
BRONJ (28). It is therefore urgent to reduce the BRONJ risk associated with these otherwise
effective medications for pathological osteolysis.

The mechanism of BRONJ is not yet fully understood (29). BRONJ exclusively occurs in
the jawbone, which uniquely associated with the gingival oral barrier tissue with one of the most
active barrier immunities (30). The presence of N-BP on the jawbone is shared by all BRONJ
patients (31) and it is conceivable that jawbone N-BP may uniquely interact with oral barrier
immunity. The major challenge has been the difficulty in separating the systemic and oral effects
of N-BP therapy, which has severely hindered the elucidation of the mechanisms of BRONJ.

N-BP chemisorption to bone results from its high affinity to hydroxyapatite (HAp), the
mineral component of bone (32). Recent investigations in our laboratories revealed that pre-
adsorbed N-BPs can be displaced from HAp by exposure to a second N-BP/BP in aqueous buffer
both in vitro and in vivo (33, 34). This led us to envision a new experimental model based on the
selective removal of pharmacologically active legacy N-BP locally from jawbone by intra-oral
application of a second BP with low pharmacological potency (lpBP). Herein, we describe a
liposome-based deformable nanoscale vesicle (DNV) formulation for topical trans-oral mucosa
delivery of an lpBP to the underlying jawbone. Here we report its effect in attenuating N-BP-
induced oral lesions using a murine BRONJ model and the elucidation of the mechanism
involved in this pathology.
RESULTS

Hydroxymethylene diphosphonate (HMDP: hydroxymethylene bisphosphonate) was selected as the lpBP

N-BPs such as zoledronate (ZOL) act on osteoclasts by inhibiting farnesyl diphosphate synthase (FPPS), thus interfering with the mevalonate pathway and protein prenylation (35). The nitrogen-containing side chain of N-BPs, an essential pharmacophore in all potent N-BP anti-resorptive drugs, is absent in hydroxymethylene diphosphonate (HMDP) which however retains an α-OH group together with two phosphonate groups, conferring high affinity to HAp (Figure 1A). Thus, we postulated that HMDP in solution might be able to decrease the amount of legacy N-BP at the bone surface by a competitive displacement mechanism as we reported (33, 34). ZOL IV injection to mice increased femur trabecular bone volume over the saline vehicle solution injection control, whereas HMDP IV injection with the same dose did not alter the bone architecture (Figure 1B and 1C). The lack of pharmacological effect of HMDP in vivo was consistent with our previous study using a standard in vitro prenylation assay (34) and confirmed HMDP to be an lpBP.

Next, we tested the ability of HMDP to displace the pre-adsorbed N-BP from bone mineral surface. Synthetic apatite (carbonate apatite)-coated culture wells were pre-treated with fluorescently tagged ZOL (FAM-ZOL: 10 µM). After thorough washing to remove non-chemisorbed FAM-ZOL, the wells were challenged by application of HMDP (10 µM in Milli-Q treated pure water: MQW). FAM-ZOL on the synthetic apatite was reduced by half (Figure 1D), and the wash solution contained a signal of dissociated FAM-ZOL (Figure 1E). After the second HMDP application, the FAM-ZOL on the synthetic apatite was further reduced (Figure 1D). This
experiment confirmed that a chemisorbed N-BP could be displaced by repeated applications of HMDP (Figure 1D, 1E).

We further examined the effect of competitive removal of ZOL on osteoclastic bone resorption in vitro. ZOL chemisorbed on synthetic apatite inhibited osteoclastic bone resorption in vitro as expected, which was measured by the decreased resorption pit area created by RAW 264.7-derived osteoclasts (Figure 1F). A single application of HMDP increased the pit area, albeit at no statistical significance. When HMDP was applied twice, the resorption pit size significantly increased and reached the level of ZOL-untreated blank wells (Figure 1F).

Although the FAM-ZOL experiment suggested that ZOL would not completely be removed after two applications of HMDP, osteoclastic activity was restored to a nearly normal level as ZOL-untreated control group (Figure 1F). We postulate that there may be a threshold bioavailable concentration of N-BP causing osteoclast abnormality. Therefore, our goal need not to be complete removal of legacy N-BP from the jawbone, but rather to decrease the local N-BP concentration below a BRONJ-triggering threshold.

Preparation of the HMDP-deformable nano-scale vesicle (DNV) formulation

In our previous proof-of-concept experiment, HMDP directly injected into mouse palatal gingiva in ZOL-pretreated mice prior to the maxillary first molar extraction was shown to prevent the development of BRONJ (34). BRONJ lesions exhibit ulcerative gingival tissue exposing necrotic jawbone. Because injection into pliable oral tissue will be challenging in some cases, and is a more focal delivery, we designed a formulation of HMDP enabling the compound to penetrate through the oral mucosa epithelial layer, making topical delivery to the jawbone possible. Liposomes have often been used as a drug carrier for controlled delivery to enhance
drug concentrations in targeted tissues and to achieve therapeutic effects using minimum drug
doses (36). Deformable nano-scale vesicles (DNV) comprise a modified liposome drug carrier
(Figure 2A) synthesized by a controlled microfluidics system, which due to nanovesicle
deformability allows penetration through the keratinized epithelial layer of skin (37).

HMDP-DNV contains HMDP in the aqueous core and lipid layers formed by 1,2-
dioleoyloxy-3-(trimethylammonium)propane-sulfate (DOTAP), diphosphatidylcholine (DPPC),
cholesterol (CH) and Span80 (15%v/v) (Figure 2A). Span80 is a surfactant that generates DNV
deformability. HMDP-DNV was manufactured through a synchronized stringent pipeline for
microfluidic synthesis, followed by dialysis and freeze-drying (Figure 2B).

DNV containing a far-red fluorophore (Alexa Fluor 647)-labeled ZOL (AF647-ZOL)
(38) (BioVinc LLC, Pasadena, CA) was manufactured (Figure 2C) to examine trans-oral mucosa
drug delivery in vivo. We also synthesized “non-deformable” nanoscale vesicle (nDNV) without
Span80 for use as a control.

The BP containing DNV sample was evaluated by particle size and surface zeta potential
(Figure 1D), which are reported to play important roles in liposome drug delivery behavior (39,
40). The optimal size for this purpose has been shown to be between 100 to 200 nm (37). After
passing through the gingival and oral mucosa epithelial layers, DNV is expected to deliver
HMDP to the bone mineral surface, which is negatively charged in isotonic solution (41). The
binding constants $K_b$ of clinical N-BPs to HAP and their effect when adsorbed at the HAP
surface on the zeta potential have been previously investigated (32, 42). At pH 7.4, the zeta
potential of HAP is about – 4 mV and essentially unchanged by adsorbed ZOL. However, HAP
binding etidronate, which is similar in structure to HMDP and like HMDP, lacks a positively
charged side chain, showed a negative zeta potential (more negative surface charge). Therefore,
cationic DNV with a positive surface potential (between +20 to +40 mV) was selected to target N-BP-chemisorbed jawbone to counteract the latter effect.

The trans-epithelial drug delivery of mouse palatal gingiva was designed to apply HMDP-DNV for 1 h. During application, the mouse palate was covered by a custom-made oral appliance using dental resin to protect from licking and accidental swallowing (Figure 2E). In the initial study, lyophilized AF647-ZOL-DNV was dissolved in MQW at different concentrations (25 µM, 75µM and 200 µM) and 3 µl of the AF647-ZOL-DNV solution was applied to the mouse palatal gingival tissue. Forty-eight hours later, euthanized mouse skulls including the palatal alveolar bone were examined for fluorescent signal (Figure 2F). AF647 fluorescent signal intensity at the maxillary bone increased with up to an applied concentration of 75 µM of AF647-ZOL and then reached a plateau.

We then prepared AF647-ZOL-DNV and AF647-ZOL-nDNV formulations, which were reconstituted in MQW or 20% polyethylene glycol (PEG). The AF647-ZOL-DNV (75 µM) reconstituted in MQW revealed the highest fluorescent signal, indicating successful AF647-ZOL delivery to the jawbone in vivo (Figure 2G). This study also demonstrated that AF647-ZOL-nDNV (75 µM) was less effective in trans-oral mucosa delivery of AF647-ZOL to the jawbone.

Development of a new mouse model: HMDP-DNV applications prior to dentoalveolar procedures in ZOL-treated mice

Female mice aged 8 to 10 weeks old were treated by a bolus ZOL intravenous (IV) injection (500 µg/Kg) from the retro-orbital venous plexus. Control mice received a vehicle solution IV injection. One week after the ZOL or vehicle injection, the maxillary left first molar
was extracted (Figure 3A). Extraction wound healing of the control group was uneventful, and the open gingival wound was mostly closed 1 week after the tooth extraction. By contrast, the tooth extraction wound of the ZOL group was delayed, which was evidenced by a gingival open wound until 4 weeks (Figure 3B).

The clinical definition of BRONJ is an unhealed oral wound with the exposed jawbone or the development of fistula reaching to the jawbone surface (Figure 3C). AAOMS defined MRONJ including BRONJ as the nonhealing oral wound for 8 weeks in human patients (14) to differentiate the normal oral wound healing such as tooth extraction, which would be healed within this period. The tooth extraction wound in mice heals in 3 weeks, which is much faster (43, 44). However, we found that the conventional mouse chaw pellets often delayed the tooth extraction wound healing due to food impaction in the extraction socket (34). Refining the mouse tooth extraction model by feeding soft gel diet after tooth extraction for 1 week, we found that the tooth extraction wound was clinically closed as early as 1 week (34). Using this refined mouse tooth extraction model, the BRONJ is assessed as open oral wound for 1 week or longer after tooth extraction or later. The gross clinical observation is accomplished by using standardized oral photographs. The prevalence of open oral wound is expressed as the percent of animals with unhealed wound in each group at a given healing time.

After tooth extraction, the bony socket undergoes a sequential wound healing resulting in bone regeneration. Micro-CT is a well-established radiographic method suitable for small animal models. We have established a quantitative method to measure the bone volume in the mouse tooth extraction socket using Micro-CT images (Figure 3-figure supplement 1). BRONJ delayed the bone regeneration process and often represented as empty tooth extraction socket in ZOL pretreated mice (Figure 3D). A recent article reported the diagnostic value of radiographs for
BRONJ (45), which exhibited radiographic features with unhealed empty extraction sockets and inflammatory lesion (Figure 3E).

The development of osteonecrosis is the hallmark of BRONJ histopathology. In the mouse model, the necropsy of harvested maxillary tissues was conducted. The tooth extraction site of ZOL-injected mice exhibited a large area of osteonecrosis defined by a cluster of empty osteocytic lacunae. The gingival connective tissue adjacent to the necrotic bone showed dense inflammatory cell infiltration associated with epithelial hyperplasia (Figure 3F). The bone biopsy specimens obtained from human BRONJ patients demonstrated the empty osteocytic lacunae as the definitive sign of osteonecrosis and associated with epithelial hyperplasia (Figure 3G), reported as pseudoepitheliomatous hyperplasia (46).

Using the mouse BRONJ model, the effect of topical application of HMDP-DNV was examined to determine whether it would modulate the BRONJ symptoms. In this study, Empty-DNV, HMDP in MQW (1.5 pmol/100 µM) or HMDP-DNV in MQW (1.5 pmol/100 µM) were applied topically to the palatal tissue of mice pretreated with ZOL IV injection prior to the maxillary left first molar extraction (Figure 3H). One time application of Empty-DNV, HMDP and HMDP-DNV did not affect the delayed tooth extraction wound healing in Micro-CT imaging and necropsy histological analyses (Figure 3-figure supplement 2). Therefore, we increased the HMDP dose by twice topical applications prior to the tooth extraction. After 2x topical applications, the HMDP-DNV treated group showed increased bone regeneration at the equivalent level of the no ZOL-pretreated control group, whereas applications of Empty-DNV and HMDP alone had no significant effect (Figure 3I).

Histological examination of the ‘HMDP-DNV 2x’-treated group showed normal extraction wound healing. The ‘Empty-DNV 2x’ and ‘HMDP alone 2x’-treated groups revealed
extensive alveolar bone osteonecrosis, characteristic of a BRONJ lesion (Figure 3J). The area of
osteonecrosis in the maxillary alveolar bone in the ‘HMDP-DNV 2x’ application group was
significantly prevented (Figure 3J). Dentoalveolar surgeries such as tooth extraction have been
reported as a risk factor to induced ONJ in patients with a history of N-BP therapy (14). The
results suggest that the twice repeated applications of HMDP-DNV prior to dentoalveolar
surgery (tooth extraction) prevented the development of BRONJ.

Targeted removal of ZOL by HMDP-DNV treatment from nascent BRONJ lesions
accelerated disease resolution in mice

BRONJ lesion in mice was defined as unhealed wound 1 week after tooth extraction in
ZOL-injected mice, showing delayed gingival wound closure and exposed alveolar bone (Figure
3B). Mouse maxillary tissues were harvested 1, 2 and 4 weeks after tooth extraction in the
untreated BRONJ group (Figure 4A). The incidence of BRONJ lesion sustained at 83.3% and
50.0% at 2 weeks and 4 weeks after tooth extraction (Figure 4B). Twice repeated HMDP-DNV
topical treatments were administered to the BRONJ lesion after 1 week since tooth extraction
(Figure 4A). Intra-oral examination revealed that the gingival wound was clinically closed in all
mice at 2 weeks after tooth extraction and further improved at 4 weeks in the topical HMDP-
DNV treatment group (Figure 4B). The area of open wound (Figure 4C) and the area of gingival
swelling (Figure 4D) were significantly reduced by HMDP-DNV treatment.

Micro-CT imaging demonstrated the impaired bone regeneration in the extraction socket
and extended osteolysis in BRONJ mice. By contrast, bone regeneration in the extraction socket
was significantly improved by the HMDP-DNV treatment (Figure 4E). The osteonecrosis area of
maxillary alveolar bone at the tooth extraction site in the untreated ZOL-injected mice
progressively increased; however, the HMDP-DNV treated group attenuated the expansion of the osteonecrosis area at 2 weeks of extraction healing, which was significantly decreased at 4 weeks (Figure 4F).

Micro-CT analysis of femur bones revealed increased trabecular bone volume and decreased inter-trabecular bone space in the ZOL-injected mice (Figure 1B, 1C), as expected as the anti-resorptive effect of ZOL. Intra-oral topical application of HMDP-DNV did not alter the femur bone trabecular architecture, which exhibited typical phenotypes of the ZOL treatment (Figure 4G). These results indicate that the treatment efficacy of HMDP-DNV was limited to the jawbone without modulating the antiresorptive effect of legacy ZOL in the other skeletal system.

Histological characterization of BRONJ lesion and the effect of HMDP-DNV treatment

The maxillary specimens were decalcified and processed for paraffine embedded histological sections. Histological evaluations of mouse tooth extraction wound showed delayed bone healing and epithelial hyperplasia leading to the exposure of jawbone in untreated ZOL-pretreated mice. Specimens harvested 4 weeks after tooth extraction exhibited the development of fistula formed by epithelial hyperplasia reaching to the alveolar bone and a lesion of pustule. More notably, inflammatory cell infiltration to the gingival connective tissue was localized on the necrotic jawbone in the untreated mice and in the empty extraction socket (Figure 5A). These histological observations appeared to be consistent with BRONJ lesion in humans. By contrast, the contralateral unwounded side of untreated ZOL-pretreated mice was free from any noticeable pathology.
The tooth extraction site of the HMDP-DNV treated mice showed the establishment of epithelial continuation and bone regeneration in the bonny socket (Figure 5A). It appeared that inflammatory reaction was subsided or less dense than the untreated lesion, and the necrotic jawbone appeared to be actively removed by bone resorption (Figure 5B). At 4 weeks after tooth extraction, the tooth extraction wound was seen well healed (Figure 5A) and the inflammatory reaction was resolved (Figure 5B). Furthermore, these specimens showed the loss of alveolar bone height adjacent to the tooth extraction socket, but the remaining bone contained only a small osteonecrosis area (Figure 5B).

It has been well established that tooth extraction induces not only bone regeneration and remodeling by osteoclasts in the bony socket but also a unique set of osteoclasts on the surface of alveolar bone (47). Histologically, osteoclasts are distinct as multinuclear large cells adhered to the bone lacunae. In addition, the presence of osteoclasts was confirmed by Cathepsin K (Ctsk) immunohistochemistry (48). The present study found that Ctsk-positive multinuclear cells were relatively small and flattened in untreated ZOL-injected mice. These osteoclasts were adhered on shallow bone lacunae not only near the tooth extraction socket (Zone A) but also on the alveolar bone surface under the palatine neuro-vascular complex and dense inflammatory cell infiltrate (Zone B) (Figure 5C). In some specimens, Ctsk-positive multinuclear cells were found in the gingival connective tissue away from the bone surface.

In contrast, the HMDP-DNV treated group showed large multinuclear Ctsk-positive cells located in the deep bony lacunae. Most osteoclasts in the HMDP-DNV treated group were clustered near the tooth extraction socket (Zone A), whereas only a few osteoclasts were found in Zone B (Figure 5C).
The number of osteoclasts was counted on the palatal surface of maxillary alveolar bone. Because the size of maxillary alveolar bone was consistent in the histological sections, the raw osteoclast number was presented (Figure 5D). The mean osteoclast number of the untreated ZOL-injected group remained high at 2 weeks and 4 weeks after tooth extraction. The HMDP-DNV treated group showed the progressively decreased osteoclast numbers and at 4 weeks after tooth extraction, almost no osteoclasts were found (Figure 5D). The appearance of osteoclasts in Zone A and Zone B was different in the HMDP-DNV treated group that osteoclasts were predominantly found in Zone A (Figure 5E).

**Oral barrier immune reaction examined by single cell RNA sequencing**

To determine the effect of HMDP-DNV treatment on the oral barrier immunity, we performed the single cell RNA sequencing of dissociated cells from gingival tissue (Figure 6A). The gingival oral barrier cells were composed of T lymphocyte and B lymphocyte and myeloid cells (Figure 6B). The fraction of T cells in the untreated BRONJ mice contained Cd8a+ cytotoxic T cells and the upregulated expression of T cell costimulatory molecule CD27 (Figure 6C), suggesting the presence of mature CD4+ and CD8+ T cell function (49). Furthermore, a small but distinct expression of Il17f indicated the differentiation of highly pro-inflammatory Th17 cells (50) in the BRONJ lesion. The oral barrier cells from HMDP-DNV treated mice did not show these pro-inflammatory signatures. Instead, it was noted that the expression of Foxp3 in their T cell fraction (Figure 6C), suggesting the presence of regulatory T cells potentially mediating inflammation resolution (51) and wound healing (52).

Similarly, Cd14+Lyz2+ myeloid cells in the untreated BRONJ lesion showed the expression of Cd68 and Itgam (Figure 6D), suggesting the involvement of macrophages in the
innate immune system contributing to the pro-inflammatory condition (53). The attenuation or suppression of these pro-inflammatory functions was suggested in the HMDP-DNV treated oral barrier tissue. The histopathological evaluation of human BRONJ biopsy samples (Figure 6E) as well as necropsy specimens from rodent BRONJ models (54, 55) reported the association with a large cluster of neutrophils.

**HMDP-DNV treatment induced myeloid-derived suppressor cell gene signature in the oral barrier tissue**

The myeloid cell fraction of the scRNA-seq data were further divided into neutrophils and macrophages (Figure 7A). It was striking that neutrophils in oral barrier tissue of the HMDP-DNV treated group lacked the expression of TREM1 (Figure 7B), which would trigger innate immune activation (56, 57). Furthermore, the expression of pro-inflammatory cytokines, *Il1a*, *Il1b* and *Tnf* was nearly negated in neutrophils and macrophages by the HMDP-DNV treatment (Figure 7C).

It was found that neutrophils as well as macrophages from oral barrier tissue of the HMDP-DNV treated group expressed *Arg1* and *Arg2* (Figure 7D), suggesting the stimulation of an anti-inflammatory reaction (58) and tissue regeneration/remodeling (59). *Arg*+ myeloid cells have also been known as myeloid-derived suppressor cells (MDSC) that negatively regulate the pro-inflammatory reaction and induce vascular development to support tissue regeneration and wound healing (60, 61). MDSC are diverse population of monocytic and granulocytic myeloid cells that are recently shown to express a common set of signature genes (62). We analyzed the scRNA seq data for the gene signature of MDSC. Macrophage and neutrophil myeloid cells
isolated from the oral barrier tissue of the HMDP-DNV treated group expressed all of the evaluated MDSC signature genes, including anti-inflammatory cytokines (Figure 7D).

Taken together, the selective removal of ZOL by topical HMDP-DNV treatment from the jawbone prevented the progressive increase of osteonecrosis, normalized osteoclastic activity, and attenuated acute and chronic inflammation, ultimately leading to healing and resolution of the BRONJ lesion (Figure 7E).
The pathological mechanism of BRONJ has not been well established. This side effect of N-BPs selectively affects jawbone in the oral cavity. The unique features of the jawbone include its proximity to the oral immune barrier and frequent osteoclastogenesis caused by dentoalveolar infection, inflammation, and wounding (30, 63). We have hypothesized that the presence of N-BP on the jawbone is a critical causal factor, which interfaces the unique oral environment. To address this hypothesis, the present study applied HMDP to remove the legacy N-BP (Figure 1). HMDP is a primary chemical component of Tc-99m-HMDP, a widely used diagnostic agent in Single Photon Emission Computed Tomography (SPECT) imaging. It is well established for its safety, and the selectivity of HMDP for SPECT imaging of abnormal bone metabolic sites (64) has been demonstrated. A recent clinical report indicated that Tc-99m-HMDP also binds efficiently to MRONJ lesions in humans (65). In view of these facts, HMDP was selected and encapsulated in DNVs (HMDP-DNV) to prepare a new topical formulation (Figure 2).

Intra-oral administration of this therapy demonstrated wound closure and radiographic extraction socket bone healing with micro-CT in mice receiving the twice repeated HMDP-DNV topical application and demonstrated a rapid BRONJ resolution (Figure 3 and 4). In our mouse model, BRONJ-like lesion was observed only at the tooth extraction area and the contralateral unwounded side did not show any noticeable abnormality, suggesting that the dentoalveolar surgery played an important role in the mouse BRONJ lesion. A recent multicenter retrospective survey confirmed that dentoalveolar surgery and tooth extraction increased the risk of BRONJ (66).

N-BP adsorbed on bone mineral stays relatively firmly until bone resorption by osteoclasts, and active bone resorption may release N-BP from bone surface to the local
environment. Tooth extraction induces not only bone remodeling in the bony socket but also unusual osteoclastogenesis on the external surface of alveolar bone (47). The present study demonstrated the oral barrier inflammation of ZOL-pretreated mice, which was densely localized adjacent to the alveolar bone surface (Figure 5). It is highly conceivable that tooth extraction-induced osteoclastic activity on the alveolar bone surface could release N-BP to the oral environment. It has been reported that N-BP mediates M1 macrophage polarization (67) and impairs neutrophil function (68). The single cell RNA sequencing analysis in the present study suggested the gene expression signature of proinflammatory myeloid cells, which was reversed to anti-inflammatory phenotypes by the HMDP-DNV treatment (Figs. 6 and 7).

In addition, the present study highlighted the pathological role of released N-BP for prolonged oral inflammation in the BRONJ lesion and the HMDP-DNV treatment induced MDSC phenotype of macrophages and neutrophils (Figure 7). ZOL treatment was shown to decrease MDSC in mammary tumor-bearing female mice (69) and to decrease tumor-associated macrophages (TAM) in mesothelioma-bearing female mice (70). MDSC and TAM are important mediators of tumor-induced immunosuppression and the inhibition of MDSC accumulation with N-BP improves the host anti-tumor response in breast cancer (69) and pancreatic adenocarcinoma (71). MDSC has been highlighted in the tumor environment as pathologically activated neutrophils and monocytes with a detrimental role of enhancing tumor growth and metastasis (72). The present study demonstrated the clear gene signature of MDSC in the gingival oral barrier tissue after HMDP-DNV-derived removal of ZOL from the jawbone (Figure 7). The HMDP-DNV treatment converted from the pro-inflammatory to anti-inflammatory phenotype. The transient presence of M2 macrophages and MDSC during wound healing protects from over-reactive immune responses (73). The reduction of gingival swelling (Figure
4) and in the resolution of chronic gingival inflammation (Figure 5) by HMDP-DNV treatment may lead to tissue repair promotion and the reestablishment of homeostasis.

The present study demonstrated that osteoclasts on the alveolar bone surface in ZOL-pretreated mice were found to form abnormally shallow bone resorption lacunae and the HMDP-DNV treatment normalized the osteoclastic activity generating large resorption lacunae (Figure 5). The osteonecrosis area of the HMDP-DNV-treated BRONJ mice did not increase compared to the osteonecrosis area before the treatment (Figure 4); however, the necrotic bone appeared to be removed by 4 weeks after tooth extraction. The treatment effect of HMDP-DNV was not to revitalize the necrotic bone. The normalized osteoclasts by HMDP-DNV treatment might resorb the necrotic bone. A case series of 25 patients described that the natural resolution of BRONJ was associated with necrotic bone sequestration and debridement leading to primary closure of the exposed bone (74). It is also possible that HMDP-DNV treatment facilitated the necrotic bone sequestration between 2 weeks and 4 weeks after tooth extraction.

Recently, osteoclasts have been found to maintain the ability to secrete a set of anti-inflammatory cytokines (75, 76). N-BPs such as ZOL altered osteoclasts to increase the secretion of pro-inflammatory cytokines (76). Furthermore, osteoclasts are found to be an efficient feeder cells supporting natural killer cells in an ex vivo system (77). A cluster of osteoclasts induced by dentoalveolar infection and surgery may play a critical role in supporting and regulating the oral barrier immunity (78). Denosumab has been used in the same indications as N-BP through a different pharmacological mechanism; but denosumab-related ONJ (DRONJ) demonstrated similar clinical and radiographic symptoms (79). We postulate that the lack of viable osteoclasts as a local immune coordinator may cause the dysregulation of oral barrier immunity leading to the development of BRONJ and DRONJ.
It must be noted that not all patients exposed to anti-resorptive medications develop MRONJ. In fact, its incidence is quite small. Therefore, other confounding factors may be involved in the pathogenesis of MRONJ. Clinical case studies proposed an increased risk of BRONJ in patients with autoimmune diseases such as rheumatoid arthritis (80), autoimmune hepatitis (81), and Sjogren syndrome (82, 83). N-BP-treated rodent models combined with experimental rheumatoid arthritis (84) or concurrent dexamethasone treatment (85) developed exacerbated BRONJ lesion supporting the susceptibility of compromised systemic immunity. However, a study of larger BRONJ cohort did not show a clear relationship (86). Autoimmune diseases are often treated with immunosuppressive agents such as glucocorticoids, which might affect the susceptibility of BRONJ (87, 88). Glucocorticoids are widely used in combination therapies for multiple myeloma and breast cancer involving use of N-BP and other anti-resorptive drugs which are implicated in MRONJ (85).

The systemic and local confounding factors certainly play a significant role in the disease severity of BRONJ. However, the present studies of topical oral administration of HMDP-DNV demonstrated that targeted removal of ZOL from the jawbone was the single most effective therapy to prevent and accelerate the resolution of BRONJ. We further underline that the local intra-oral application of HMDP-DNV did not affect the distant skeletal system and thus the pathological mechanism of BRONJ is likely localized within the oral tissue. These observations support our hypothesis that above the threshold dose level of oral N-BP holds the critical key for the pathological mechanism to develop BRONJ. Finally, the work reported here establishes the basis for the development of this novel treatment as an effective prophylactic and therapeutic method for ZOL-induced BRONJ, and possibly for BRONJ associated with other N-BPs.
MATERIALS AND METHODS

Chemical reagents

Zoledronate (ZOL) was acquired from UCLA Medical Center Pharmacy (Reclast®, Novartis, Basel, Switzerland). Fluorescent-tagged ZOLs (FAM-ZOL and AF647-ZOL) were obtained from BioVinc LLC (Pasadena, CA). HMDP (oxidronic acid; hydroxymethylene-1,1-bis(phosphonic acid) was acquired from Aroz Technology (Cincinnati, US; Cat # BP-1026) as the disodium salt and characterized by $^1$H NMR (D$_2$O, 600 MHz): δ 3.78 (t, J = 16 Hz), $^{31}$P NMR (D$_2$O, 243 MHz): δ 14.90 (s) and by elemental analysis: calculated for CH$_4$O$_7$P$_2$Na$_2$, 4.98% C, 1.92% H; found 4.97% C, 1.91% H (>99% purity). DOTAP (1,2-dioleoyloxy-3-(trimethylammonium)propane-sulfate), DPPC (diposphatidylcholine), CH (cholesterol) and the nonionic surfactant Span 80 were acquired from Sigma-Aldrich (St. Louis, MO).

The effect of ZOL and HMDP in mouse femur bone structure in vivo

Female C57BL/6J mice (n=5 per group) were anesthetized by isoflurane inhalation and 100 µl of ZOL (40 nmol), HMDP (40 nmol) or saline vehicle solution was injected to retro-orbital venous plexus (44, 54). Three weeks after the injection, mice were euthanized and femur bones were harvested for micro-CT imaging (µCT40, Scanco Medical AG, Southeastern, PA) following the standard procedure. Bone parameters were determined using the proprietary analysis program.

Competitive displacement removal of ZOL by HMDP in vitro

Cell culture wells coated with carbonate apatite (Bone resorption assay plate 24, Cosmo Bio Co. Ltd, Tokyo, Japan) were incubated with fluorescent-tagged ZOL (10 µM FAM-ZOL,) overnight at 37°C, 2% CO$_2$, followed by 3 washes with Milli-Q treated pure water (MQW) for
10 min each. The FAM-ZOL coated wells were then incubated with 10 µM HMDP in MQW (n=6) or MQW (N=3) for 2 hr at 37°C, 2% CO₂ followed by 3 washes with MQW. One group of HMDP treated wells (n=3) were treated by the second application of 10 µM HMDP. Other wells were treated by MQW. After washes, the FAM fluorescent signal of each well was evaluated (IVIS Lumina II, PerkinElmer, Waltham, MA): excitation 465 nm; and emission filter: GFP (38). The region of interest was set to the well size and the fluorescent signal was measured. Separately, all wash solutions were subjected to fluorescent signal evaluation.

**Osteoclast resorption pit formation assay in vitro**

Cell culture wells coated with carbonate apatite were incubated with ZOL (10 µM in MQW) for overnight at 37°C, 2% CO₂ followed by extensive washes. ZOL-preincubated wells were treated with 10 µM HMDP once (n=3) or twice (n=3) as described above. Control wells were treated with MQW (n=3). After the final wash, culture medium (MEM containing 10% fetal bovine serum and 1% antibiotics mix) was added to all wells. Then, RAW274.1 cells (2.5x10⁴ cells per well) were inoculated to each well in culture medium supplemented by mouse recombinant receptor activator of nuclear kappa-B ligand (RANKL; Sigma-Aldrich) (100 ng/ml) and incubated at 37°C, 2% CO₂. The culture medium was changed after 3 days. The resorption pits were photographed after 6 days of incubation after the cells were removed with 0.25% Tripsin. The total area of resorption pit was measured using a Java-based image processing program (ImageJ, NIH, Bethesda, MD).

**Synthesis of HMDP-DNV and AF647-ZOL-DNV**

Fabrication of the DNV formulation containing BP compounds followed the published method (37). Briefly, cationic DOTAP lipid with DPPC and CH were dissolved in a 10 mM chloroform solution at a 5:3:2 volume ratio and the mixture was allowed to dryness. The dried
The aqueous solution was comprised of deionized water and the BP compound (12.9 µM) and filtered (0.2 µm). The organic and aqueous solutions were used for DNV synthesis using microfluidics reactor (Syrris, Royston, UK) with a 26 µL reactor chip at 1000 µL/min organic stream and 5000 µL/min aqueous stream, followed by dialysis to remove the non-encapsulated BP compound and free lipids and then freeze-dry processes. Physical characteristics such as the size and zeta potential were determined by a Malvern Zetasizer (Nano-ZS: Malvern, Worcestershire, UK) following the manufacturer’s protocol.

BP encapsulation efficiency (EE) was calculated as percent of AF647-ZOL amount in the DNV relative to the initial AF647-ZOL amount used in the synthesis. An aliquot of the lyophilized AF647-ZOL-DNV or AF647-ZOL-nDNV was lysed by adding acetonitrile. Next, samples were shaken for half an hour, then 350 µL of water was added to solubilize the compounds and samples were centrifuged for ten minutes at 22000xg. The supernatant was collected for HPLC. A standard curve was created using the original 12.9 µM stock solution of each of the compounds by HPLC for AF647-ZOL-DNV. Using the equation for the line of best fit for the signals received, we inputted the average signal given by the samples post-dialysis to obtain their concentration. It should be noted that the amount/concentration of all BP-DNV or BP-nDNV discussed in the following in vitro and in vivo studies were all calculated based on the encapsulated BPs in the DNV/nDNV formulation.

**LCMS analysis method for the encapsulation efficiency measurement of HMDP-DNV:**

**Sample preparation**
Five hundred (500) µL of ice-cold LCMS grade ACN was added to vial containing lyophilized HMDP-DNVs. The mixture was vortexed for 3 mins and sonicated for 20 mins to break the DNVs. This sequence was repeated once. Next, 2 µL LCMS grade water was added to each vial and the mixture was vortexed for 10 secs and centrifuged at 10,000 rpm for 30 mins. Following the centrifugation, 1.5 µL of the supernatant was collected and separated from the pellet. A 5 mg/µL HMDP stock solution was prepared from the supernatant using maximum theoretical encapsulation value of 100 mg/vial. From the stock, 200 µL, 100 µL, and 40 µL corresponding to 1000 ng, 500 ng, and 200 ng HMDP respectively, were added to 1-dram glass vials. To each vial was added 200 µL of 500 ng/µL etidronate disodium solution as internal standard (IS). The samples were lyophilized. 500 µL trimethyl orthoacetate (TMOA) and 150 µL acetic acid was added to the lyophilized samples, and the mixture was heated at 100°C for 1h for derivatization. The solvents were evaporated under a stream of air. The residue was dissolved in 1 µL LCMS water. The samples were filtered, transferred to 1 µL HPLC vials, and the HMDP content was analyzed using LCMS. For generating standard curve, standard solutions were prepared by adding known concentrations of HMDP to 1-dram glass vials along with 200 µL of 500 ng/µL etidronate disodium as IS. The solutions were lyophilized, derivatized, and processed as mentioned above.

The LCMS system consisted of Waters Aquity H-class UPLC coupled with Waters Xevo G2-XS QTOF equipped with Masslynx 4.2 software (Waters, Massachusetts, USA). Chromatography was performed on Syncronis™ C18 Column (2.1 x 50 mM, particle size 1.7 mM, Thermo Scientific) using water (mobile phase A) and ACN (mobile phase B) at 30°C and 0.4 µL/min. The chromatography gradient was 0 - 0.3 mins (0% B), 0.3 - 0.8 mins (0 - 100% B), 0.8 - 1.6 mins (100% B), 1.6 - 2 mins (100 - 0% B), and 2 - 3.5 mins (0% B). The QTOF was
operated in positive ion mode monitoring m/z transitions for methylated derivates of HMDP (313 > 161) and etidronate (327 > 267). Measurements of at least three different concentrations with 3x – 5x replicating tests at each concentration were performed, and the final EE value is calculated as the average value of these tests. Using the theoretical concentration of the samples after synthesis, we were able to calculate the encapsulation efficiency of the liposomes as 23%, which was verified (+/- 5%) by direct analysis of the unmodified HMDP-DNV formulation by a $^{31}$P NMR method (Supplemental file).

**Estimation of HMDP content in final DNV preparation by $^{31}$P NMR**

To verify the HMDP content of HMDP-DNV, we conducted an independent analysis based on $^{31}$P NMR. Using the LCMS-based approach, the encapsulation efficiency (EE) was calculated as percent of HMDP amount in the DNV relative to the initial HMDP amount used in the synthesis. By this method, the final EE value for HMDP-DNV averaged to 23% which corresponds to a concentration of 3.0 µM for HMDP encapsulated by the liposome. Unlike the LCMS-based approach, $^{31}$P NMR analysis does not require chemical derivatization of the HMDP. Thus, an aliquot of the HMDP-DNV formulation was used directly as the sample. The average integration of the $^{31}$P NMR signal for an accurately weighed sample of HMDP in D$_2$O was determined relative to the signal of an external reference of known concentration (analytically pure MDP (methylenebisphosphonic acid, disodium salt)). The same system was then used to determine the concentration of HMDP in the HMDP-DNV formulation, in D$_2$O. The value obtained by the latter method was 2.7 µM, which agreed within error with the value of 3.0 µM from the LCMS analysis, giving an encapsulation efficiency of 23% for a starting HMDP concentration of 12.9 µM in the HMDP-DNV synthesis.
Topical drug application to the mouse palatal mucosa and AF647-ZOL-DNV delivery to the jawbone

We designed a topical application protocol for delivery of BP-DNV to the maxillary jawbone through palatal mucosa. Mouse oral appliances were fabricated using clear orthodontic dental resin to fit to the entire mouse palatal mucosa surface between the molar teeth. Mice were anesthetized by isoflurane inhalation and placed on a surgical bed to open the mandible. Three (3) µl of AF647-ZOL-DNV or AF647-ZOL-nDNV reconstituted in MQW or 20% polyethylene glycol solution was pipetted to the palatal mucosa and covered by an oral appliance. The mandible was closed with a bite block and the animal was placed in the anesthesia chamber. Mice were kept anesthetized for 1 hour and the oral appliance was then removed. After 24-48 h, mouse maxillae were harvested and the AF647 fluorescent signal was measured by an imaging reader (LAS-3000, Fujifilm Corp, Tokyo, Japan). While AF647-ZOL-DNV assisted the trans-oral epithelial delivery, some AF647-ZOL-DNV appeared to be metabolized in the oral cavity and the released AF647-ZOL was adsorbed to the exposed tooth surface or the enamel structure. Thus, we set the region of interest (ROI) only on the palatal bone excluding the tooth structure.

Induction and characterization of BRONJ lesion in the mouse model

Female C57Bl6/J mice (8 to 10 weeks of age) received a bolus intravenous injection of ZOL (500 µg/Kg in saline solution) or vehicle saline solution from the retro-orbital venous plexus. It was important to prevent traumatic tooth extraction, which caused a serious confounding factor for sound tooth extraction wound healing. The younger mice at 8-10 wks allowed consistent atraumatic tooth extraction in our study (44, 54). One (1) week later, the maxillary left first molar was extracted under general anesthesia by isoflurane inhalation (44, 54). After tooth extraction, all mice were fed gel food (DietGel Recovery, Clear H₂O, Portland,
ME) for 1 week and then switched to regular mouse pellet chaw. After switching to the regular pellet chaw, all mice were briefly anesthetized once a week and food and debris impaction was removed. Mice were euthanized at 1 week, 2 weeks or 4 weeks after tooth extraction and femur bones and the maxillary tissues including the extraction wound were harvested after being photographed. The buffered formalin-fixed maxillary tissues and femur bones underwent Micro-CT imaging. The maxillary tissues were demineralized by 1 M EDTA in 4°C and prepared for paraffin-embedded histological sections stained with hematoxylin and eosin. Digitized histological images were examined, and the area of osteonecrosis was measured using the ImageJ program. The mouse oral lesion, Micro-CT radiography and histopathology were compared to clinical information from human BRONJ patients.

Topical application of HMDP-DNV to the mouse BRONJ model prior to tooth extraction

ZOL-injected mice were treated with topical application of Empty-DNV, HMDP in MQW (1.5 pmol/100 µM) or HMDP-DNV in MQW (1.5 pmol/100 µM) to the palatal oral mucosa. The topical formulation was applied 1 time (Figure S3A) or 2 times (Figure 3H) in a week and then the maxillary left first molar was extracted. Two (2) weeks after tooth extraction, mouse maxillae and femur bones were harvested. The development of BRONJ lesion was examined as described above.

Topical application of HMDP-DNV to the established mouse BRONJ lesion

ZOL-injected mice underwent a maxillary left first molar extraction. Delayed healing and BRONJ-like jawbone exposure were confirmed 1 week after tooth extraction. HMDP-DNV in 3 µl MQW (1.5 pmol/100 µM) was topically applied to the BRONJ lesion and surrounding gingival tissue. This treatment was applied 2 times total in a week. The maxillary tissues and
femur bones were harvested at 2 weeks and 4 weeks after tooth extraction and the BRONJ lesion was characterized as described above.

**Histological characterization of osteoclasts**

In the histological sections stained by hematoxylin and eosin (H&E), osteoclasts were identified as cells containing at least 3 nuclei and localized on the bone surface. Selected histological sections were further subjected to immunohistochemical staining with anti-cathepsin K (Ctsk) antibody (Cat# ab19027, Abcam, Waltham MA). Sections were deparaffinized and treated with an antigen retrieval procedure using citrate buffer (pH 6.0) and high heat for 2 min followed by a conventional blocking process. After the incubation with the anti-Ctsk rabbit polyclonal primary antibody, the anti-rabbit IgG secondary antibody was used with 3,3’-diaminobenzidine chromogenic reaction. The sections were then counter stained with hematoxylin.

Using both H&E stained and Ctsk immunohistochemical sections, the number of osteoclasts was counted on the palatal surface of maxillary alveolar bone of the tooth extraction side. When the gingival connective tissue was significantly pealed from the bone surface, the sections were excluded. Next, the palatal surface of maxillary bone was equally divided into 2 zones: Zone A was the lateral zone toward the edge of tooth extraction socket; and Zone B was the medial zone toward the mid-palatine suture, typically under the palatal neuro-vascular structure of gingiva. Because the mouse maxillary bone was highly consistent for the palatal surface size, the osteoclast number was not normalized by the bone surface area and was presented as raw data.

**Single cell RNA sequencing of gingival oral barrier immune cells**
After the HMDP-DNV treatment of the BRONJ mice, the gingival tissue from the tooth extraction side of the palate was harvested (n=4). The gingival tissue of untreated BRONJ mice was also harvested (n=4). Gingival tissues were cut into 1-mm pieces and placed in digestion buffer containing 1 mg/ml collagenase II (Life Technologies, Grand Island, NY), 10 units/ml DNase I (Sigma-Aldrich) and 1% bovine serum albumin (BSA; Sigma-Aldrich) in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) for 20 min at 37°C on a 150-rpm shaker. The tissues were passed through a 70µm cell strainer. The collected cells were pelleted at 1,500 rpm for 10 min at 4°C and resuspended in phosphate buffered saline (PBS; Life Technologies) that was supplemented with 0.04% BSA (Cell suspension A).

The remaining tissues were further incubated in 0.25% trypsin (Life Technologies) and 10 units/ml DNase I for 30 min at 37 °C on a 150 rpm shaker. The combined trypsin-released cells and collagenase II-released cells were counted and the equal number of cells (2,000~3,000) from each animal were combined in the group for single cell RNA sequencing (10X Genomics, San Francisco, CA). The Cell Ranger output of single cell RNA sequencing data were analyzed using R-program (Seurat, https://satijalab.org/seurat/).

**Statistical analysis**

The mean and standard deviation were used to describe the data. The Turkey test was used to analyze multiple samples and statistical significance was considered to be achieved if p<0.05.

**Study approval**

All animal experiments were performed at UCLA. All the protocols for animal experiments were approved by the UCLA Animal Research Committee (ARC# 1997-136) and
followed the Public Health Service Policy for the Humane Care and Use of Laboratory Animals and the UCLA Animal Care and Use Training Manual guidelines. The C57Bl/6J mice (Jackson Laboratory) were used in this study. Animals consumed gel or regular food for rodents and water ad libitum and were maintained in regular housing conditions with a 12-hour-light/dark cycles at the Division of Laboratory Animal Medicine at UCLA.

The human BRONJ biopsy data were collected under the approval by the UCLA Institutional Review Board (IRB #17-000721). Clinical demonstration of human BRONJ was obtained from patients of UCLA School of Dentistry clinic with the general consent for educational use. The information was not part of investigator-initiated research.

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Data availability

All data generated or analyzed during this study are included in the manuscript and supporting file. Single cell RNA sequencing data have been deposited in GEO under accession code GSE193110.


Figure Legends

Figure 1. Competitive equilibrium-based dissociation of N-BP by low potency BP (lpBP).

(A) Chemical structures (shown as the tetraacids) of N-BP, zoledronate (ZOL) and hydroxymethylene diphosphonate (HMDP). (B) ZOL but not HMDP affects mouse femur trabecular bone architecture. Mice received a bolus intravenous injection of 100µl ZOL (40 nmol), HMDP (40 nmol) or vehicle saline (0.9% NaCl) solutions. Femurs (n=4 per group) were harvested 3 weeks after the IV injection and subjected to Micro-CT imaging. (C) HMDP did not affect the femur trabecular bone micro-architecture, whereas ZOL increased bone volume over total volume (BV/TV), connectivity density (ConnD) and trabecular thickness (Tb.Th). Trabecular number (Tb.N) and trabecular separation (Tb.Sp) measurements were not affected by ZOL. (Figure 1 source data 1) (D) *In vitro* demonstration of competitive displacement of legacy N-BP. Synthetic apatite-coated wells pre-incubated with 10 µM FAM-ZOL were washed with MilliQ-treated pure water (MQW), then treated with 10 µM HMDP once (1x) or twice (2x) (n=3 per group). The FAM fluorescent signal measurement indicated significant reduction of the FAM-ZOL amount on the synthetic apatite. (Figure 1 source data 2) (E) Fluorescent signal measurement of the wash solutions from the *in vitro* experiment in (D) demonstrated removal of FAM-ZOL by the HMDP treatments (arrows). (Figure 1 source data 3) (F) *In vitro* osteoclastic pit formation assay. Synthetic apatite coated wells were pre-incubated with 10 µM ZOL followed by 10 µM HMDP treatment 1x or 2x (n=3 per group). RAW274.1 cells (2.5x10⁴ cells per well) were then inoculated to each well in culture medium supplemented by mouse recombinant receptor activator of nuclear kappa-B ligand (RANKL). The areas of resorption pits generated by osteoclasts derived from...
RAW 264.7 cells were measured after 6 days of incubation and cell removal. Twice repeated HMDP treatments restored normal in vitro resorption pit formation. (Figure 1 source data 4) In D and F, the graphs show the mean and standard deviation (n=3 per group), and the Turkey test was used to analyze multiple samples. The statistical significance was determined at p<0.05. In E, the graphs show the mean and standard deviation (n=3 per group), and the Turkey test was used to analyze multiple samples within each time point. The statistical significance was determined at p<0.05.

**Figure 2. Manufacturing and trans-oral mucosal penetration evaluation of HMDP-DNV and AF647-ZOL-DNV.** (A) Diagram of DNV, a liposome derivative. (B) Flow diagram of micro-fluidics based DNV synthesis. (C) Confocal laser scanning microscopy of AF647-ZOL-DNV. Approximately 100-200 nm DNV particles exhibited an AF647 signal. (D) Characterization of DNV formulations. (E) Protocol for intra-oral topical application to mouse palatal tissue. The reconstituted DNV solution in MQW (3 µl) was topically applied to the palatal gingiva between maxillary molar teeth and covered by a custom-made oral appliance fabricated with auto-polymerizing dental resin. After 1 hour, the oral appliance was removed. (F) After topical application of AF647-ZOL-DNV, mouse maxillary bones were harvested and AF647 fluorescence was measured. The AF647 fluorescent signal from the maxillary bone region of interest (ROI) increased with up to 75 µM AF647-ZOL in DNV applied and then reached a plateau. (Figure 2 source data 1) (G) AF647-ZOL-DNV and AF647-ZOL in non-deformable formulation (AF647-ZOL-nDNV) were reconstituted in either MQW or 20% polyethylene glycol (PEG). AF647-ZOL-DNV in MQW most efficiently delivered the drug to the maxillary bone
through trans-oral mucosal route. (Figure 2 source data 2) In F and G, the graphs show the mean and standard deviation (n=6 per group), and the Turkey test was used to analyze multiple samples. The statistical significance was determined at p<0.05.

Figure 3. Disease phenotypes of mouse and human BRONJ lesion. (A) Experimental protocol for inducing a BRONJ lesion in mice. Mice received a bolus IV injection of ZOL (40 nmol/500 µg/Kg) or vehicle saline solution were subjected to maxillary left first molar extraction. (B) Intra-oral photographs depicted that mouse pretreated with ZOL IV injection exhibited delayed wound healing with sustained open wound (black arrows) and gingival swelling (white arrows). (C) Human BRONJ lesions with open tooth extraction wound (black arrows) and gingival swelling (white arrows). (D) Micro-CT images of mouse maxilla depicted that delayed bone regeneration in the mesial, buccal and palatal root extraction sockets (white arrows) in ZOL-pretreated mice, a sign of BRONJ symptoms. (E) Radiographic demonstration of unhealed tooth extraction of human BRONJ. (F) Histological evaluation of mouse BRONJ lesion with osteonecrosis (dotted line; Nec), gingival inflammation (Inf) and epithelial hyperplasia reaching to the necrotic bone (black arrows). (G) A biopsy specimen of human BRONJ lesion with osteonecrosis (Nec) and gingival epithelial hyperplasia (black arrows). (H) A time course experimental diagram of HMDP-DNV application. All mice received ZOL IV injection. Empty-DNV, HMDP in MQW (HMDP alone) (1.5 pmol/100 µM) or HMDP-DNV in MQW (1.5 pmol/100 µM) was topically applied to the palatal gingiva prior to the maxillary left first molar extraction. (I) Micro-CT evaluation of tooth extraction socket, which remained empty (white arrows) in the groups treated with Empty-DNV or HMDP alone. Two
topical applications of HMDP-DNV prior to the tooth extraction significantly increased tooth extraction socket bone regeneration compared to Empty-DNV and HMDP alone. (Figure 3 source data 1) (J) Histological evaluation. Two topical applications of HMDP-DNV prior to the tooth extraction reduced the development of osteonecrosis compared to the treatment of Empty-DNV and HMDP alone, which remained to exhibit BRONJ phenotype. (Figure 3 source data 2) In I and J, the graphs show the mean and standard deviation (n=3 per untreated control group and n=6 per experimental group), and the Turkey test was used to analyze multiple samples. The statistical significance was determined at p<0.05.

**Figure 3-figure supplement 1. Mouse BRONJ model.** (A) Time course protocol to develop BRONJ in the mouse. Mice received a bolus IV injection of ZOL (500 µg/Kg) or vehicle saline solution and were subjected to maxillary left first molar extraction. The maxillary tissue was harvested 1, 2 and 4 weeks after the tooth extraction, photographed and scanned by micro-CT. (B) Three-dimensional reconstruction of micro-CT images showed mesial, buccal and palatal root extraction sockets. (C) Quantitative BV/TV measurement of extraction sockets of mesial, buccal and palatal roots of micro-CT images demonstrated a significant delay of bone regeneration in ZOL-treated mice, a signifier of BRONJ.

**Figure 3-figure supplement 2. Single Intra-oral topical application of HMDP-DNV to ZOL-treated mice prior to tooth extraction did not prevent development of a BRONJ lesion.** (A) Time course experimental diagram. All mice received a ZOL IV injection.
HMDP-DNV, Empty-DNV or HMDP in saline solution (HMDP alone) was topically applied to the palatal gingiva prior to the maxillary left first molar extraction. Two weeks after tooth extraction, the maxillary tissue was harvested and subjected to micro-CT imaging and demineralized paraffine embedded histological section preparation. (B) A single application of HMDP-DNV, Empty-DNV or HMDP alone did not produce any improvement of bone regeneration as measured by BV/TV of micro-CT images and reduction of histological osteonecrosis area. (C) Histological evaluation also indicated that the single application of HMDP-DNV, Empty-DNV and HMDP alone did not show reduction of histological osteonecrosis area.

Figure 4. HMDP-DNV topical application to the established BRONJ lesion in mice accelerated the disease resolution. (A) The time course experimental protocol. After ZOL IV injection and tooth extraction, a BRONJ lesion developed, which was treated by two topical applications of HMDP-DNV in MQW (1.5 pmol/100 µM). (B) Intra-oral photographs depicted the unhealed tooth extraction wound (black arrows) with gingival swelling (dotted line) in significant percentage of animals in untreated mice group (‘No Tx’ group) from 2 weeks to 4 weeks after tooth extraction. By contrast, all HMDP-DNV-treated mice exhibited a closed wound in week 2, which was well healed in 4 weeks after tooth extraction. (C) The wound opening of untreated mice remained 4 weeks after tooth extraction, while HMDP-DNV topical treatment minimized the wound opening 2 weeks after tooth extraction. (Figure 4 source data 1) (D) The gingival swelling area was also decreased by HMDP-DNV topical treatment. (Figure 4 source data 2) (E) Micro-CT analysis showed the delayed bone regeneration in the extraction sockets (white arrows) of
untreated ZOL-injected mice. However, HMDP-DNV-treated ZOL-injected mice accelerated the extraction socket bone regeneration, which was further remodeled to generate bone marrow trabecular structure (white arrow heads). The bone volume/total volume of the extraction socket showed the early bone regeneration in the group of HMDP-DNV treatment. (Figure 4 source data 3) (F) The histological osteonecrosis area progressively increased in the BRONJ lesion. HMDP-DNV topical treatment halted the osteonecrosis area increase at 2 weeks and decreased at 4 weeks after tooth extraction. (Figure 4 source data 4) (G) The topical application of HMDP-DNV did not affect distant skeletal tissue in femurs. (Figure 4 source data 5) In C, D, E, F and G, the graphs show the mean and standard deviation (n=5~6 per group), and the Turkey test was used to analyze multiple samples. The statistical significance was determined at p<0.05.

**Figure 5. HMDP-DNV topical application normalized tooth extraction wound healing of ZOL-pretreated mice.** (A) Histological evaluation depicted BRONJ lesion at the tooth extraction site of the untreated ZOL-injected mice (No Tx) exhibiting that the abnormal epithelial hyperplasia (E. Hyp) extending to the necrotic alveolar bone (Nec) appeared to facilitate the sustained open wound, the uneven bone regeneration in the tooth extraction sockets (Soc) and localized infiltration of inflammatory cells (Inf) on the alveolar bone surface. The abnormal wound healing pattern was also observed at 4 weeks after tooth extraction with fistula formation by epithelial hyperplasia reaching to the necrotic bone and a localized pustule lesion (Pus). The unwounded side of ZOL-injected mice did not show any abnormality of remaining tooth (Tooth), alveolar bone (Bone) and overlining gingival tissue (Gingiva). After the HMDP-DNV topical treatment, gingival wound was
found closed with more diffused inflammation (Inf) and a sign of bone resorption (while arrowheads) was depicted on the surface of alveola bone. The tooth extraction socket (Soc) showed bone regeneration at week 2, which was further remodeled and matured at week 4. **(B)** High magnification histology of 2 weeks after tooth extraction demonstrated the dense inflammatory cell infiltration (Inf) in the gingival connective tissue and osteonecrosis area (Nec) in untreated ZOL-injected mice (No Tx). The HMDP-DNV treatment attenuated the inflammatory cell infiltration and increased signs of osteoclastic bone resorption (white arrowheads). At 4 weeks after tooth extraction, the untreated mice continued to show dense inflammatory cell infiltration and osteonecrosis. However, mice with the HMDP-DNV treatment showed subsided inflammation and the minimized osteonecrosis area likely due to bone resorption, which appeared to result in alveolar bone loss (dotted white line and arrow) as compared to the regenerated bone in the tooth extraction socket (Soc). **(C)** Cathepsin K (Ctsk) immune-stained osteoclasts on the alveolar bone surface of the untreated ZOL-injected mice were observed not only at the proximal area (Zone A) of the tooth extraction socket (Soc) but also under the palatine neuro-vascular complex (Zone B) with inflammation (Inf). Characteristically, these Ctsk-positive osteoclasts were small and flattened (black arrowheads). In some specimens, Ctsk-positive cells were observed away from the bone surface (small arrowheads). The HMDP-DNV-treated mice showed large Ctsk-positive osteoclasts in deep bone lacunae adjacent to the tooth extraction socket (Zone A). **(D)** Total number of osteoclasts defined as Ctsk-positive multi-nuclear cells on the alveolar bone surface. (Figure 5 source data 1) **(E)** Osteoclasts in Zone A and Zone B were separately counted. (Figure 5 source data 2) In D and E, the graphs show the mean and standard deviation (n=5 per group), and the
Turkey test was used to analyze multiple samples. The statistical significance was determined at p<0.05.

Figure 6. Single cell RNA sequencing of gingival cells of untreated and HMDP-DNV-treated ZOL-injected mice. (A) Two weeks after tooth extraction, gingival tissue adjacent to the tooth extraction site was harvested for cell dissociation followed by single cell RNA-sequencing. (B) Using signature gene expression, myeloid cells, T cells and B cells were identified. (C) T cell-related gene expression indicated the presence of Cd8a+ cytotoxic, Cd27+ matured T cells derived from mouse BRONJ gingiva. Il17f expression phenotype was decreased by HMDP-DNV treatment, which increased Foxp3 Treg phenotype. (D) Macrophage-related genes demonstrated an increase in M1 macrophages in untreated BRONJ gingiva, which was decreased by HMDP-DNV treatment. (E) Human BRONJ biopsy samples showing that necrotic bones (Nec) were associated with a large cluster of neutrophils (black arrows).

Figure 7. Characterization of myeloid immune cells. (A) The myeloid cell fraction of scRNA-seq was further divided into neutrophils and macrophages. (B) Myeloid immune cells identified by Trem1 demonstrated a significant decrease of neutrophils by HMDP-DNV treatment. (C) Pro-inflammatory cytokines Il1a, Il1b, and Tnf were highly expressed in macrophages and neutrophils from BRONJ gingiva. (D) Gingival macrophage and neutrophil myeloid cells after HMDP-DNV treatment expressed the multiple signature genes of the myeloid-derived suppressor cell (MDSC): Arg1, Arg2, CD84, Wfdc17,
*Ifitm1, Ifitm2, Clec4d, Clec4e, Ctsd, Cstdc4* (from (62)) or M2 macrophage phenotypes *Arg1, Arg2*: as well as anti-inflammatory cytokines *Il1f9* and *Il10*. (E) A hypothetical model of BRONJ.
Table 1. Estimated MRONJ case numbers in the US based on MRONJ incidence for the major underlying diseases*

<table>
<thead>
<tr>
<th>Underlying Diseases</th>
<th>New Cases in the US (Year)</th>
<th>MRONJ incidence</th>
<th>Estimated MRONJ Cases (Year)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple Myeloma</td>
<td>34,920**</td>
<td>5.16% (89)</td>
<td>1,802</td>
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<tr>
<td>Breast Cancer</td>
<td>330,840***</td>
<td>2.09% (89)</td>
<td>6,915</td>
</tr>
<tr>
<td>Prostate Cancer</td>
<td>248,530**</td>
<td>3.80% (89)</td>
<td>9,444</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>1,800,000****</td>
<td>0.01% (90)</td>
<td>18,000</td>
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<tr>
<td><strong>Estimated Annual Incidents of MRONJ</strong></td>
<td><strong>36,161</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Based on an assumption that all these patients were treated by anti-resorptive medications.

**American Cancer Society. *Cancer Facts & Figures 2021.* Atlanta, Ga: American Cancer Society; 2021


A

- 7 days
HMDP-DNV
IV (ZOL)
- 2 days
HMDP-DNV
Tooth Extraction
Tissue Collection
0
Empty-DNV
IV (ZOL)
DNV (empty)
Tooth Extraction
Tissue Collection
2 weeks
HMDP alone
IV (ZOL)
HMDP
Tooth Extraction
Tissue Collection

B

1 X application

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BV/TV (%)</th>
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<tbody>
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<td>HMDP-DNV</td>
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</tr>
<tr>
<td>Empty-DNV</td>
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<tr>
<td>HMDP alone</td>
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</table>

C

1 X application

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Osteonecrosis Area (%)</th>
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</thead>
<tbody>
<tr>
<td>HMDP-DNV</td>
<td></td>
</tr>
<tr>
<td>Empty-DNV</td>
<td></td>
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<tr>
<td>HMDP alone</td>
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